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Biodegradation of N,N-dimethylacetamide by *Rhodococcus* sp. strain B83 isolated from the rhizosphere of pagoda tree

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ABSTRACT

The biodegradation characteristic and potential metabolic pathway for removal of environmental N,N-dimethylacetamide (DMAC) by *Rhodococcus* sp. strain B83 was studied. *Rhodococcus* sp. strain B83 was isolated from the rhizosphere of a pagoda tree and proved capable of utilizing DMAC as sole source of carbon and nitrogen. Batch culture studies showed that strain B83 could tolerate up to 25 g/L DMAC and showed distinct growth on possible catabolic intermediates except for acetate. The nitrogen balance analysis revealed that approximately 71% of the initial nitrogen was converted to organic nitrogen. DMAC degradation has led to accumulation of acetate and organic nitrogen, meanwhile traces of nitrate and ammonia was build-up but without nitrite. The growth of strain B83 could be inhibited by adding exogenous acetate. By means of the assay of enzymatic degradation of DMAC, several catabolic intermediates at different intervals were observed and identified. Based on the results obtained from culture solution and enzymatic degradation assay, a detailed pathway is proposed for DMAC biodegradation.

Introduction

N,N-dimethylacetamide (DMAC) is a water-miscible solvent being widely used for agrochemicals, pharmaceuticals, fine chemicals, man-made fibers, industrial coatings, films, paint strippers and other applications. Based on emission factors, a large amount of DMAC is released into the environment during manufacturing and application, even after recovery treatment. Considering its wide presence, toxicity, and slow rate of degradation, DMAC may have an adverse effect on the environment, public health and welfare. DMAC is readily absorbed by humans after oral, dermal, or inhalation exposure (Wexler, 2014). Some evidences reported that human DMAC exposure mainly causes liver toxicity, skin irritation, headache, in appetence, fatigue, and hepatic damage (Princivalle et al., 2010; Gescher and Threadgill, 1990; Menegola et al., 1999; Oechtering et al., 2006). Recently, DMAC has been listed as a chemical known to cause reproductive toxicity (Wexler, 2014).

Due to its adverse effects, many attempts have been made to develop technologies to remove DMAC contamination from industrial effluents. The chemical and physical technologies mainly include sorptive microextraction of titania and zirconia hollow fibers (Li et al., 2009), photocatalytic oxidation (Ge et al., 2012; Zhang et al., 2009), adsorption (Takatsuji and Yoshida, 1998) and internal microelectrolysis (Liu et al., 2012). The trickle-bed air biofilter (TBAB) has been proven to be an effective process treating DMAC waste gas, more than 90%

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and 80% DMAC removal efficiencies were achieved for influent DMAC loadings below 20.2 and 34.5 g/(m^3 hr), respectively (Lu et al., 2001). The biological degradation is undoubtedly a non-destructive, low-cost and environmentally-friendly technology as compared to chemical and physical methods and is worth exploring for removing DMAC contamination from industrial effluents. However, there has been no available report about the isolation of the bacteria and pure cultures, which can utilize DMAC as the sole carbon and energy source. Furthermore, the catabolic pathway involved in the biodegradation process of DMAC has not been elucidated. Therefore, it is important to screen DMAC-degrading bacterial strains from relevant environments, and to investigate the degradation characterization and metabolites for acquiring a more comprehensive understanding of the metabolic pathway of pollutant in the environment and for an effective bioremediation strategy of DMAC.

In this study we report isolation of a bacterial strain *Rhodococcus* sp. strain B83, which is capable of utilizing DMAC as the sole carbon, nitrogen and energy source. As one of the important bacteria which were extensively studied for degradation of organic pollutants, *Rhodococcus* sp. was distributed in various environments such as the water and soil. Therefore, this species have important research value and broad application prospects in environmental pollution treatment and bioremediation (Khan et al., 2013; Shen et al., 2009; Lu et al., 2009; Grishko et al., 2013; Homklin et al., 2012; Bajaj et al., 2014; Yi et al., 2011). The growth characteristics and degradation characteristics of *Rhodococcus* sp. strain B83 degrading DMAC are investigated in this communication. A detailed pathway is proposed for the biodegradation of DMAC by strain B83 based on the identification of the catabolic intermediates.

1. Materials and methods

1.1. Source of strain, chemicals, media and culture conditions

Soil samples were collected from the rhizosphere of a pagoda tree in Guanzhong region (Shaanxi, China). DMAC (99.5%) was purchased from the TJFCH Corporation (Tianjin, China). N-methylacetamide (99%) and acetamide (99%) were purchased from Aladdin Corporation (Shanghai, China). Unless otherwise stated, the organic solvents, media, salts and acids were purchased from various sources (Sigma, VWR and Fisher in USA or China). Beef extract-peptone medium containing filter-sterilized DMAC of different concentrations was used as enrichment medium. Minimal media (MM) were used for isolation and cultivation of DMAC-degrading bacteria; the MM without carbon and nitrogen are as follows (g/L distilled water): $Na_2HPO_4{\cdot}2H_2O$ 2.0, $MgSO_4$ 0.5, KCl 0.5, KH_2PO_4 1.0, $Fe_2(SO_4)_3{\cdot}H_2O$ 0.2, $CaCl_2{\cdot}2H_2O$ 0.1. The 100 mL MM was transferred to 250 mL Erlenmeyer flask and autoclaved at 121 °C for 20 min. Different concentrations of filter-sterilized DMAC (1–25 g/L) were added to the minimal medium as source of carbon and nitrogen.

1.2. Enrichment and isolation

For the purpose of enrichment 1 g soil sample was added to 100 mL of beef extract-peptone medium containing filter-sterilized DMAC (1 g/L); it was incubated for 5 days at 35 °C and shaker rate 120 r/min. Further enrichment was performed by transferring 10% bacterial suspension to fresh beef extract-peptone medium with the concentration of DMAC gradually increased to 10 g/L and the concentration of beef extract-peptone lowered. The isolation of DMAC-degrading bacteria was carried out by pipetting 10 mL enrichment bacterial suspension into 100 mL MM containing 5 g/L DMAC as the sole source of carbon and nitrogen for 5 days. After several repetitive inoculations of the culture, isolation was performed by serial dilution of the cultures and plating them on MM medium plates containing agar (DMAC 5 g/L). The purity of the culture was checked morphologically by microscopic observation. The strains obtained were separately inoculated in 250 mL Erlenmeyer flasks containing 100 mL of MM medium with 5 g/L DMAC for 5 days and the DMAC-degrading bacteria were found by determining the residual concentration of DMAC with high performance liquid chromatography (HPLC). The bacterial strain B83 utilizing solely DMAC as the source of carbon and nitrogen was selected for further research.

1.3. Identification

The colony morphology, cell morphology, Gram staining and other biochemical tests were carried out for the characterization of the strain as per standard procedures (Dong and Cai, 2001). Genomic DNA extraction of strain B83 was carried out using DNA isolation kit (MoBio Laboratories, USA) following manufacturer's recommendations, the concentration of extracted DNA was measured with a Nanodrop-2000 μ-spectrophotometer (Thermo Electron, USA) as per the manufacturer's instructions. The PCR amplification (Eppendorf Mastercyclerep, Germany) was carried out with the universal primers of both forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) by following procedures (Moreno et al., 2002): initial denaturation at 95°C for 5 min, denaturation at 94°C for 30 sec, renaturation at 56°C for 30 sec, and elongation at 72°C for 80 sec. In total 35 thermal cycles and the final elongation was at 72°C for 10 min (Mehdi et al., 2012). The PCR-amplified 16S ribosomal ribonucleic acid (rRNA) gene fragments were purified by agarose gel electrophoresis (1.2%, V/V), and the purified product was detected by MultiImager (Syngene GBoxEF, USA) according to the manufacturer's instructions. Nucleotide sequences of the 16S rRNA genes were determined by Shanghai Sangon Biotech Ltd. (China). The BLAST was performed by searching for similar sequences at the National Center for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignment was performed using ClustalX (Thompson et al., 1997). Phylogenetic analysis was performed though the neighbor-joining method using MEGA version 5.0 (Tamura et al., 2011; Saitou and Nei, 1987).

1.4. Growth on possible catabolic intermediates

To study the use of intermediates of DMAC degradation by the strain B83, N,N-dimethylformamide (DMF), dimethylamine, monomethylamine, N-methylacetamide, acetamide and formate were individually used as the sole carbon and nitrogen source in the minimal medium at an initial concentration of

5 g/L. Ammoniacal nitrogen (NH_4Cl) and nitrate nitrogen ($NaNO_3$) were also individually used as the sole nitrogen source in the minimal medium (adding with 10 g/L glucose as the sole carbon source) at an initial concentration of 5 g/L to test its ability of utilizing nitrogen. Acetate was used as the sole carbon in the minimal medium (adding with 5 g/L NH_4Cl or $NaNO_3$ as the sole carbon source individually) at an initial concentration of 5 g/L. Before inoculation, the pH of each group was adjusted to 7.0 using 0.5 mol/L HCl. All of culture media were incubated at 35°C with shaker rate 120 r/min. The increase of the biomass and the pH in the culture medium were monitored periodically.

1.5. Biodegradation of different concentrations of DMAC

A ring slant culture of B83 for 3 days was separately transferred into fresh MM contained different initial concentrations of DMAC (1, 5, 10, 15, 25 g/L), incubated at 35°C with shaker rate 120 r/min. The biomass and pH were monitored periodically. Samples of the culture taken at 12 hr intervals was centrifuged at 8000 r/min and filtered through 0.45 μm millipore filters, and the concentration of residual DMAC in the culture media was determined by HPLC (LC-2010, JASCO, Japan).

1.6. Fate of nitrogen during DMAC catabolism

During intervals ammonia and nitrate were detected with different methods in order to study the terminal products of DMAC catabolized by strain B83. The ammonia was collected periodically through a self-made simple equipment and monitored by a colorimetric method. A 250 mL Erlenmeyer flask contained 100 mL of MM (10 g/L DMAC) was plugged with a sterile rubber buffer contained air pipelines of inflow and outflow. The stable inflow was 60 mL/min of sterile air and the outflow air was led in 50 mL of boronic acid (20 g/L). The volatile and residual of ammonia were monitored by using Nessler's colorimetric method (Wei et al., 2002) to find evidence of ammonia as terminal product of DMAC catabolism. The anions of the culture filtrate at different intervals were determined by using HPLC.

Nitrogen balance tested in Erlenmeyer flasks contained 100 mL MM medium with 1 g/L DMAC. The approximate nitrogen-removal rate of strain B83 was determined by subtracting the final TN concentration from the initial TN concentration. The intracellular nitrogen level was tested according to the method of Huang et al. (2015). The soluble organic nitrogen level was calculated by subtracting NO_3^--N, NO_2^--N, and NH_4^+-N from the final soluble TN concentration.

1.7. Resting cell experiment

To substantiate the data obtained from the culture, 100 mL fresh cells of mid-logarithmic growth phase ($OD_{600\ nm} \approx 0.6$) culturing in beef extract-peptone medium were harvested by centrifugation at 8000 r/min for 10 min, washed twice with pure water and resuspended with 10 mL pure water. The cell suspension was divided into two aliquots, one aliquot was heat killed by incubating on boiling water for 30 min, this aliquot was later used as the negative control. The 1 mL cell suspension withdrawn from each aliquot were individually added to a flask containing 20 mL aqueous solutions of DMAC (10 mg/L), Each flask was then incubated at 32°C with shaking at 80 r/min for about 20 hr. The samples (1 mL each) were collected by centrifugation at 8000 r/min for 20 min from both control and experimental flasks at different intervals. The concentration of residual DMAC and degradation intermediates containing an acetyl group that might present in these samples was analyzed by HPLC.

1.8. Cell disruption and catabolic intermediates analysis

Due to the peaks of degradation intermediates containing acetyl group did not appear as we expected except for the peak of DMAC in the resting cell experiment, further assay of the enzymatic degradation of DMAC was carried out, in the hope that the intermediates containing acetyl group could be found. The 100 mL fresh cells of mid-logarithmic growth phase ($OD_{600\ nm} \approx 0.6$) were harvested by centrifugation at 8000 r/min for 10 min, washed twice with pure water and resuspended with 10 mL pure water. The suspension of B83 cells in ice–water was disrupted by using an ultrasonic cell disruption apparatus (JN-02C, Guangzhou Juneng Biological Science and Technology Co. Ltd., China) at 450 W for 30 sec periods, interrupted by 30 sec intervals. In total 60 cell disruption cycles were applied. Cell debris was removed by centrifugation at 10,000 r/min for 30 min at 4°C, the supernatant thus obtained was used as the enzyme source. This supernatant was divided into two parts, one part was heat killed by incubating on boiling water for 30 min, this part was later used as the negative control. The 1 mL supernatant withdrawn from each part was individually added to a flask containing 20 mL aqueous solutions of DMAC (10 mg/L), the following operations are same as the resting cell experiment. Since the degradation intermediates contained an acetyl group the absorption spectrum at 220 nm wavelength was analyzed at different intervals by HPLC. The intermediates were identified by using ultra performance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) (Acquity TQD, Waters, Milford, MA, USA). The concentration of anions was also determined by using HPLC under different detecting conditions.

1.9. Analytical methods

The optical density (OD) of cell growth in different culture at regular intervals was determined at 600 nm using a Spectrophotometer (VIS-7220N, Beijing Beifen-Ruili Analytical Instrument Co. Ltd., China). The pH was measured in a pH meter (PHS-3C, Shanghai Precision & Scientific Instrument Co. Ltd., China). DMAC and several expected intermediates with an acetyl group were determined quantitatively by using JASCO LC-2010 HPLC equipped with UV–vis Diode array detector and C18 ODS Hypersil column (5 μm, 250 × 4.6 mm) in the reverse phase mode. The HPLC separation was carried out within 10 min using 8% CH_3OH (V/V) at injection amount of 5 μL and a flow rate of 1.0 mL/min, with the column temperature 40°C. The structure of intermediates was identified by the UPLC-ESI-MS/MS instrument (Acquity TQD, Waters, Milford, MA, USA) according to the method of Ge et al. (2012). The

anions were also determined by HPLC (JASCO LC-2010 HPLC, C18 ODS Hypersil column 250 × 4.6 mm, particle size 5 μm and ultraviolet wavelength 210 nm). A 0.1 mol $NH_4H_2PO_4$ solution was used as the solvent and flow rate of 1 mL/min with column temperature 40°C. The HPLC separation was carried out within 20 min per injection of 5 μL. The ammonia was monitored by Nesslerization (Wei et al., 2002). TN was determined according to the standard UV spectrophotometry method (DR6000, HACH, American) (Chen et al., 2012).

2. Results and discussion

2.1. Isolation and identification of DMAC biodegradation bacteria

Several bacterial strains capable of growing on MM agar plates containing 10 g/L DMAC were isolated from the soil samples. From the isolated strains the strain B83 was found capable of biodegrading DMAC completely with DMAC as the sole source of carbon and nitrogen. The cells of strain B83 were Gram positive, non-spores, short rod-shaped and demonstrate branched filaments during the early growth phase; which then cleaved into short rods during the stationary growth. Bacterium B83 cultured in 18–24 hr on MM (5 g/L DMAC) or beef extract-peptone medium agar plats formed ivory, circular, smooth and opaque colonies. The color would turn to orange after 2–4 days of incubation at 35°C. The strain was tested positive for catalase, nitrate reduction, nitrite reduction, ammonia production, nitrosation and nitrification tests, while Voges–Proskauer, Methyl Red, acetic acid oxidation and denitrification tests were negative. The 16S rRNA nucleotide sequence of strain B83 was 1436 bp long and the sequence was deposited at the EMBL databank with accession number KF990162. The 16S rRNA sequence of strain B83 clustered with the type strains obtained from NCBI (Fig. 1). Sequence comparison revealed that strain B83 has a sequence similarity of 99% with *Rhodococcus rubber* DSM 43338^T. Based on its morphological and physiological characteristics, biochemical tests and 16S rRNA gene sequence analysis, strain B83 was identified and named as *Rhodococcus* sp. strain B83.

2.2. Growth characteristic on the possible catabolic intermediates

Nitrate nitrogen served as good sources of nitrogen, excellent growth was observed when strain B83 was grown on nitrate nitrogen, final $OD_{600\ nm}$ increase reached maximum value of 2.27 in 72 hr. DMAC, acetamide, N-methylacetamide, DMF were also used as sources of carbon and nitrogen, the $OD_{600\ nm}$ increases of the cultures reached maximum of 0.88, 0.82, 0.64 and 0.53 in 120 hr, respectively. Along with the cell proliferation in the above medium culture the pH increased significantly 7.0 to 8.5 or near. Dimethylamine and monomethylamine could not be served as sources of carbon and nitrogen, poor growth was recorded and the pH value remained unchanged.

Faint growth was observed when acetate and ammoniacal nitrogen were use as sources of carbon or nitrogen, the $OD_{600\ nm}$ increases of the cultures only reached maximum of 0.21 and 0.23 in 120 hr, respectively. The pH value in medium having acetate was increased to 8.5 or near, but the pH value in medium having ammoniacal nitrogen was decreased to 2.7 or near. The result of faint growth in acetate suggested that it is hard to utilize acetate as source of carbon source for strain B83. The process that mostly influences the pH of the liquid phase should be nitrification in medium having ammoniacal nitrogen, which causes a pH decrease due to proton production (Gernaey et al., 1998; Massone et al., 1996; Ramadori et al., 1980). In contrast, the bacteria could grow well in medium

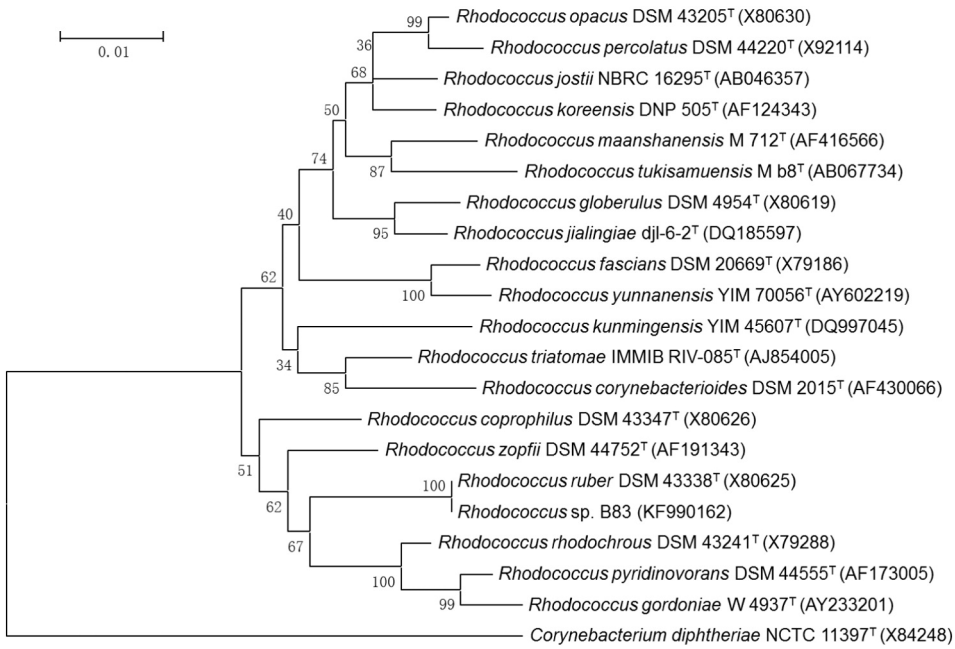

Fig. 1 – Neighbor-joining tree shows phylogenetic position of *Rhodococcus*sp. strain B83 based on the 16S rRNA sequence. rRNA: ribosomal ribonucleic acid.

with ammoniacal nitrogen through controlling the pH value in the range of neutral or slightly acidic, final $OD_{600\ nm}$ increase reached maximum value of 1.87 in 72 hr. It is suggested that ammoniacal nitrogen could serve as good source of nitrogen for strain B83.

2.3. DMAC biodegradation and accumulation of acetate and nitrate ions

The growth characteristics of B83 cultured in different initial concentration of DMAC were observed (Fig. 2a). Maximum growth of the bacterium was observed during 24–72 hr of incubation. Growth decreased beyond 15 g/L of initial DMAC. The strain showed growth even at 25 g/L although the specific growth was significantly decreased. The concentration of residual DMAC along with the increase biomass showed that DMAC was biodegraded rapidly during the logarithmic phase and utilized completely within 96 hr of incubation with initial DMAC concentration of 10 g/L. Further increase of DMAC in culture medium reduced degradation efficiency of DMAC. The degradation efficiency is 96.1% of 15 g/L and 58.8% of 25 g/L initial DMAC in 120 hr. The strain showed a surprising endurance and a remarkable ability of degradation high concentrations of DMAC. The initial concentration of DMAC had no significant effect on the pH of the culture, whereas had significant adverse effect on the growth beyond 15 g/L of initial DMAC (Fig. 2b).

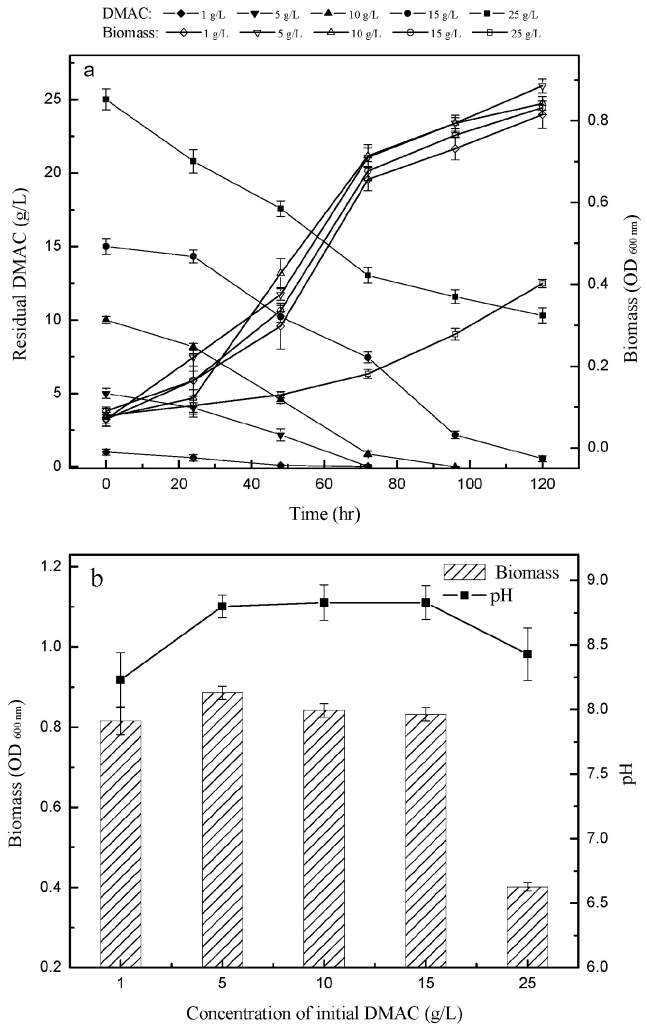

Fig. 2 – (a) Biodegradation of DMAC along with the increase biomass of *Rhodococcus*sp. strain B83 with respect to time with different concentration of initial DMAC; (b) growth characteristic of *Rhodococcus* sp. strain B83 on different initial concentration of DMAC for 120 hr. DMAC: dimethylacetamide.

During DMAC biodegradation there was no clear evidence of large accumulations of intermediate products of DMAC degradation, such as DMF, N-methyl-N-hydroxymethylacetamide, N-methylacetamide, N-hydroxymethylacetamide and acetamide. The results of HPLC indicated that there were some substances with stronger polarity (no retention in this mobile phase) in small amounts during DMAC degradation. The test results of the anions (Fig. 3) showed that many acetate ions were accumulated during DMAC degradation and only a little of nitrate ions. Fig. 4 shows the growth curve of the culture of *Rhodococcus* sp. strain B83 along with the accumulation of acetate ions at an initial DMAC concentration of 10 g/L. The logarithmic growth phase was observed at the period of 24–72 hr. After 96 hr of incubation, the DMAC was completely biodegraded in the culture filtrate. The acetate accumulated during the decline of DMAC concentration. Release of acetate ions was equivalent to 74.2% of the initial DMAC concentration, the rest was probably further transformed into some other products. The results showed that there were traces of nitrate and ammonia accumulation but without nitrite during DMAC catabolism.

2.4. Nitrogen balance during DMAC catabolism

Nitrogen balance test in the MM medium contain with 1 g/L DMAC were carried out to research the fate of nitrogen during DMAC catabolism. The results of nitrogen balance analysis showed the TN concentration was (159.5 ± 0.71) mg/L during the initial stage. At the end of the process, the concentration

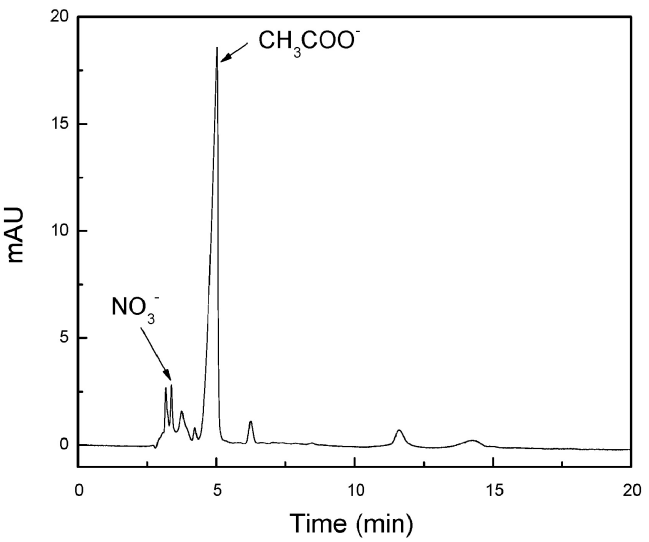

Fig. 3 – Result of the anions by using HPLC when strain B83 was cultured within 10 g/L concentration of initial DMAC for 5 days. HPLC: high performance liquid chromatography.

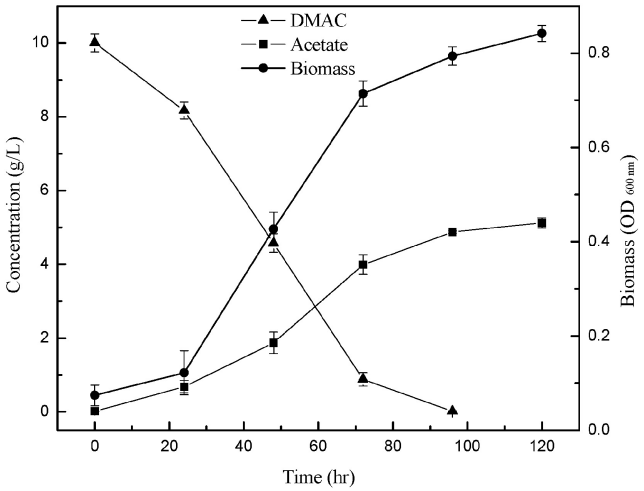

Fig. 4 – Growth and DMAC utilization along with the accumulation of acetate ion by *Rhodococcus* sp. strain B83 on 10 g/L concentration of initial DMAC.

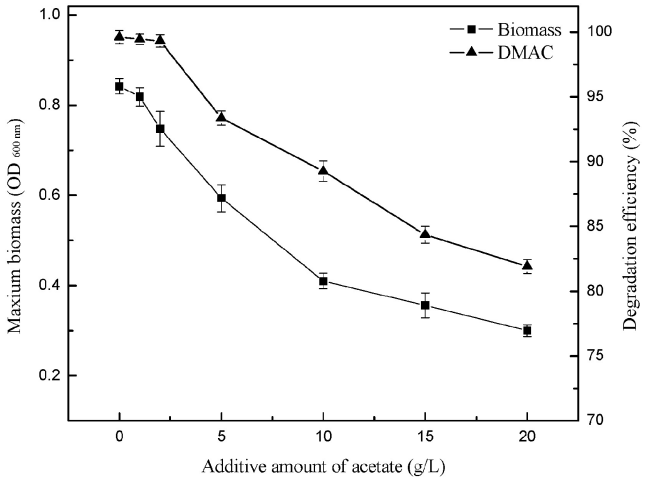

Fig. 5 – Effects of additive amount of acetate on growth and DMAC degradation efficiency, 10 g/L of initial DMAC concentration for 5 days.

of NH_4^+-N, NO_3^--N and organic nitrogen was (0.48 ± 0.14), (0.94 ± 0.31) and (113.7 ± 3.69) mg/L, respectively. And the NO_2^--N was not detected. A comparison of the initial and final TN mass in this reaction system showed that (18.26 ± 2.57)% of initial nitrogen was converted to biomass, and approximately 71% of the initial nitrogen was converted to organic nitrogen.

2.5. Effects of exogenous acetate on DMAC biodegradation

To evaluate the effects of acetate on DMAC biodegradation, fresh MM of 10 g/L initial concentration of DMAC was added to serial concentrations of acetate (1, 2, 5, 10, 15, 20 g/L). Before inoculation, the pH of each group was adjusted to 7.0 using 0.5 mol/L HCl. Fig. 5 shows the adverse effects of additive amount of acetate on growth and DMAC degradation efficiency. When the concentration of additive acetate is more than 2 g/L, the max biomass ($OD_{600\ nm}$) for 5 days decreased significantly from 0.75 to 0.3, and the degradation efficiency of DMAC for 5 days declined from 99.3% to 81.9%. The effects of other exogenous carbon substrates on the DMAC biodegradation were also researched. The addition of glucose, starch and beef extract, respectively, to the minimal salt medium with 10 g/L DMAC could significantly promote the strain's growth and degradation efficiency (data not shown). The max amount of acetate accumulation reached 5.12 g/L with the complete degradation of DMAC (10 g/L) in MM. Some microorganisms reported could excrete a large amount of acetic acid by the cells and accumulated in the culture medium, with the increase of acetic acid concentration, cell growth could be decreased and stopped even (Konstantinov et al., 1990; MacDonald and Neway, 1990; Wu et al., 1996). The results suggested that accumulation of diffusible inhibitors such as acetate may be a significant factor limiting cell growth and production expression. Acetic acid for some microorganisms has toxicity and inhibited their growth (Zhou and Gao, 2007). In the previous section, the test of growth on possible catabolic intermediates proved that it is difficult for strain B83 to utilize acetate as source of carbon source, and the DMAC degradation results showed that there had been much accumulation of acetate in the process of DMAC degradation along with the cell growth. Adding exogenous acetate could have an adverse effect on the biomass and DMAC biodegradation efficiency. The results suggested that the accumulation of high concentration acetic acid for strain B83 may be a factor limiting cell growth and DMAC biodegradation efficiency, adding exogenous acetate had greatly intensified this adverse effect.

2.6. Results of enzymatic degradation

For a better understanding of the bacterial degradation pathway of DMAC, resting cell experiments were performed with 10 mg/L concentration of DMAC. The free cells have successfully degraded DMF within 96 hr of incubation, but the test results did not show absorption peaks of intermediate products during the biodegradation of DMAC (data not shown). Therefore, further research of DMAC degradation in the crude enzyme was carried out. Fig. 6 showed the change trend of DMAC and catabolic intermediates at different intervals in crude enzymatic degradation process of 10 mg/L concentration of initial DMAC. In the phase of 3 hr, there was no obvious accumulation of some intermediates. But after that, several different metabolic products in different phases appeared, however, all of them only contained trace amounts for a short time and finally disappeared. The peaks of acetamide and N-methylacetamide were observed at the time of 5.5 hr, and disappeared with 9 hr (Fig. 6a3, a4). The proposed intermediates in DMAC biodegradation have been identified by interpretation of the mass spectrometry analysis (Fig. 7). As expected, N-methyl-N-hydroxymethylacetamide, N-methylacetamide, N-hydroxymethylacetamide and acetamide were all exited and identified. The anions present in the process of DMAC degradation on crude enzymatic degradation were also determined by HPLC. The test results showed that acetate and nitrate were produced immediately at the initial phase, and ultimately leading to complete disappearance (Fig. 6b). Quantitation of DMAC in crude enzymatic degradation showed its decrease from initial concentration of 9.84 mg/L to non-detectable amounts after 10 hr

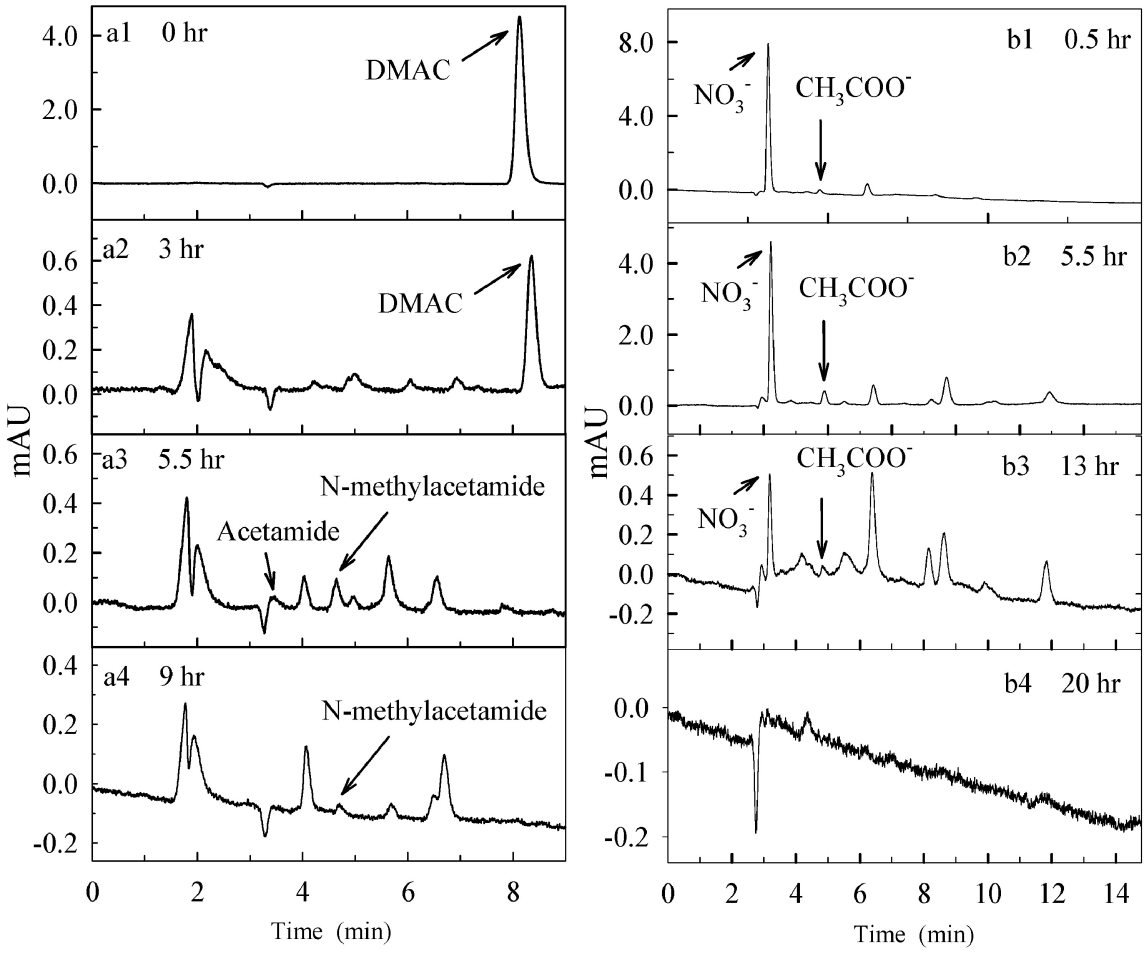

Fig. 6 – Chromatograms showing the change trend of DMAC and catabolic intermediates (a) and anions along with DMAC degradation (b) at different intervals.

of incubation (Fig. 8). The concentration of residual DMAC decreased rapidly from 9.46 to 1.03 mg/L within 1.5–4.5 hr, but slowed down afterwards, and DMAC was degraded completely within 10 hr. The nitrate concentration was immediately enlarged approximately to 0.77 mg/L within 10 min, and it was not significantly reduced within 5.5 hr. After 5.5 hr the nitrate concentration decreased slowly until complete degradation within 18 hr. The acetate was produced immediately at the initial phase, after a decline the concentration of acetate rose again. The max accumulation concentration of 8.8 mg/L of acetate appeared at the time of 3.5 hr, and then decreased until complete degradation within 20 hr. The results described in the previous section clearly show that there was about 74.2% of the acetate accumulated in the culture media, suggesting that the cells of *Rhodococcus* sp. strain B83 could not use acetate for the cells as carbon source, probably partly incorporating it with the excess acetate discharged from the cells, and the rest be further transformed into some other products.

2.7. Proposed pathway for DMAC biodegradation

Two different pathways have been proposed for the bacterial degradation of DMF (Ghisalba et al., 1985; Veeranagouda et al., 2006; Hasegawa et al., 1997; Swaroop et al., 2009). Few bacterial strains hydrolyze DMF to dimethylamine (DMA) and formate (pathway I). DMA is further converted to monomethylamine (MMA). These products are further degraded to ammonia and carbon dioxide (Veeranagouda et al., 2006; Hasegawa et al., 1997; Swaroop et al., 2009). In the second pathway (pathway II), the DMF is degraded by repeated oxidative demethylations leading to the generation of formamide, the formamide is further hydrolyzed to ammonia and formate (Ghisalba et al., 1985; Hasegawa et al., 1997). In this study, *Rhodococcus* sp. strain B83 could grow on N-methylacetamide and acetamide but not on dimethylamine and methylamine, in support of the degradation pathway, N-methyl-N-hydroxymethylacetamide, N-methylacetamide, N-hydroxymethylacetamide and acetamide are all identified in the process of DMAC enzymatic degradation. *Rhodococcus* sp. strain B83 should degrade DMAC through repeated oxidative demethylations leading to the generation of acetamide. The DMAC is firstly oxidized to N-methyl-N-hydroxymethylacetamide once it is inside the bacterial cell, then transformed into N-methylacetamide with the shedding of hydroxymethyl groups. The N-methylacetamide is further oxidized to N-hydroxymethylacetamide, and then transformed into acetamide with the shedding of hydroxymethyl groups. The

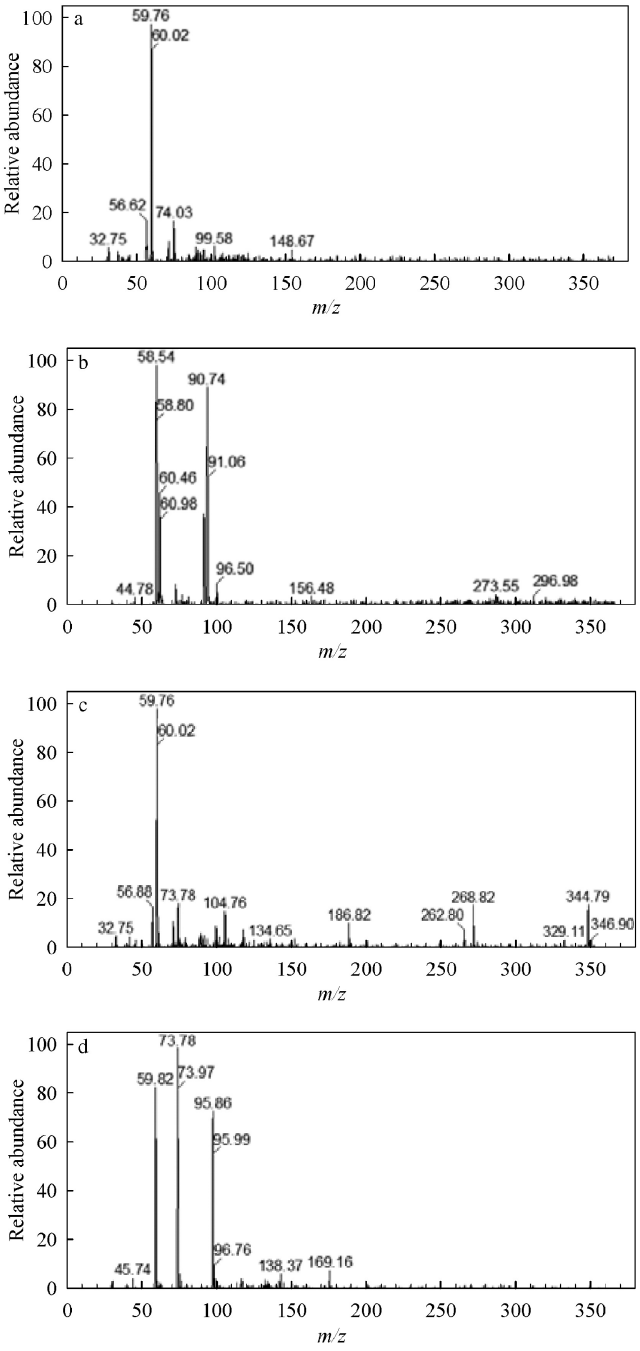

Fig. 7 – Mass spectra of the compounds of DMAC enzymatic degradation after 1.5 hr. *m/z* is 60 for acetamide (a), *m/z* is 90 for N-hydroxymethylacetamide (b), *m/z* is 104 for N-methyl-N-hydroxymethylacetamide (c) and *m/z* is 73 for N-methylacetamide (d).

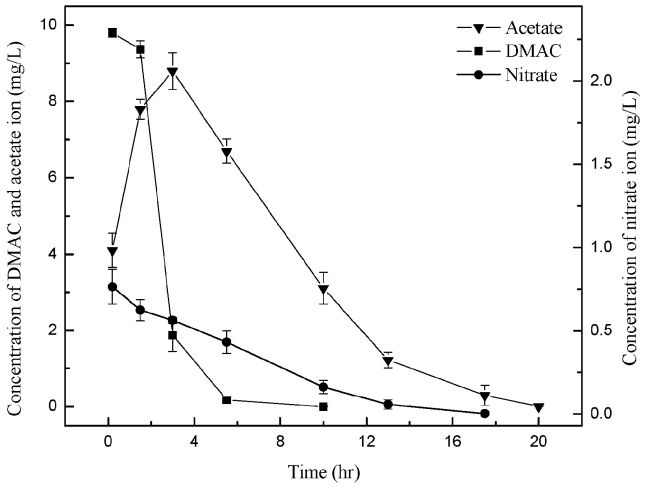

Fig. 8 – Enzymatic gradation of DMAC along with the change trends of nitrate and acetate with respect to time for 10 mg/L of initial DMAC concentration.

hydroxymethyl groups by repeated oxidative demethylations might be converted to methanol (or possibly formaldehyde) and then utilized as carbon source for the cell metabolism.

Many microorganisms are able to hydrolyse carboxylic amides and release free ammonia and carboxylic acid (Shukor et al., 2009; Minseok and Glenn, 2011). Some microorganisms are also capable of hydrolyzing acrylamide into acrylic acid and free ammonia by utilizing acrylamide as the sole source of carbon and nitrogen (Minseok and Glenn, 2011; Nawaz et al., 1992; Wampler and Ensign, 2005; Prabu and Thatheyus, 2007; Kanokhathai and Jittima, 2011; Richi et al., 2012; Thanvacharoen et al., 2012; Liu et al., 2013; Lakshmikandan et al., 2014). *Brevibacterium* sp. R312 is able to hydrolyse primary amides and hydrolyse of the secondary amide N-methylacetamide is expected based on the found acyl transfer of N-methylacetamide to hydroxylamine (Thiéry et al., 1986). Two different pathways have been proposed for the bacterial degradation of DMF, the ammonia and formate are the end products. The available literature indicates that ammonia is released as the end product of the degradation of amide compounds. In this study, the release of acetate and ammonia were all detected in the media culture of DMAC. So there's a reason to believe that acetamide could be hydrolyzed into ammonia and acetate.

The conventional system for ammonium removal by nitrifying bacteria is a two-step process in which sequential oxidation of NH_4^+-N into NO_2^--N and then NO_2^--N into NO_3^--N occurs. Ammonium removal by heterotrophic microorganisms has usually been reported to oxidize NH_4^+-N to NO_2^--N or NO_3^--N and simultaneously covert NO_2^--N or NO_3^--N to N_2O and/or N_2 (Chen et al., 2012; Khardenavis et al., 2007; Robertson et al., 1988; Zhang et al., 2003). The conclusions that the ammonia was removed but almost neither nitrite nor nitrate was found were demonstrated in some reports. Guo et al. (2013) showed that *Pseudomonas stutzeri* strain T1 was capable of conducting heterotrophic nitrification–aerobic denitrification and had both excellent nitrate and ammonium removal without nitrite build-up. Zhang et al. (2011) showed that during the whole process of ammonium removal experiment, neither nitrate nor nitrite was detected. Zhang et al. (2013) showed that ammonium was consumed by the strain SFA13 with the biodegradation of organic carbon and without nitrite or nitrate accumulation. Chen et al. (2012) showed that there were less than 2% nitrite and less than 7% nitrate accumulated, and experimental results indicate that *Rhodococcus* sp. CPZ24 utilizes ammonia or converts it to other nitrogen species. In this research, the nitrosation and

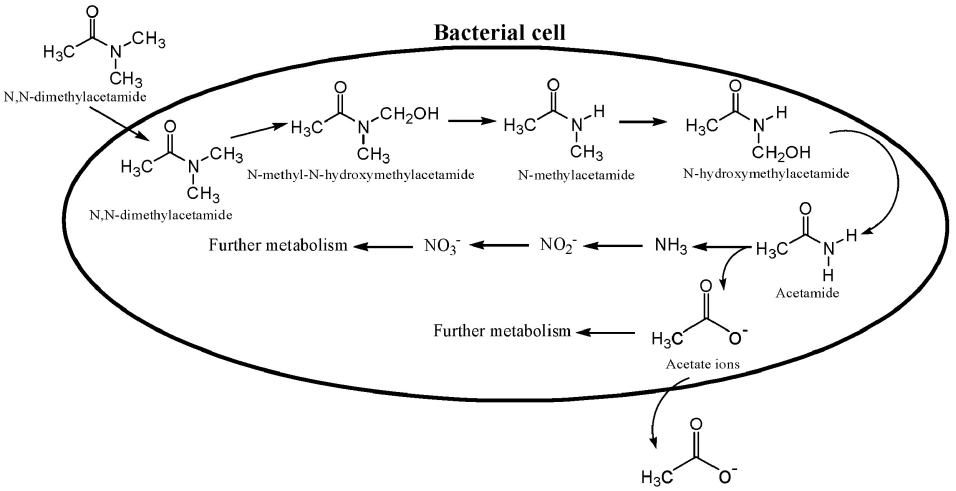

Fig. 9 – Proposed pathway for DMAC biodegradation in *Rhodococcus*sp. strain B83.

nitrification tests proved *Rhodococcus* sp. strain B83 having the ability of heterotrophic nitrification to translate the ammonia into nitrite, and also could translate the nitrite (NO_2^-) into nitrate (NO_3^-). The release of nitrate was established through assaying the products in the media culture and enzymatic degradation of DMAC, but the nitrate only contained trace amounts without nitrite build-up meanwhile. And the nitrogen balance analysis revealed that approximately 71% of the initial nitrogen was ultimately converted to organic nitrogen. According to the above analysis, a possible explication is that the ammonia hydrolyzed from the acetamide is translated into nitrate through nitrification, and the nitrate is converted to other nitrogen species. The acetate ions might be less suitable as a carbon source utilized by *Rhodococcus* sp. strain B83, and as a result, part of the acetate needs to be discharged from the cells.

By combining all the analysis results, a detailed metabolic pathway of the DMAC biodegradation by *Rhodococcus* sp. strain B83 was proposed (Fig. 9).

3. Conclusions

Rhodococcus sp. strain B83 could efficiently and completely biodegradate high concentrations of DMAC. DMAC biodegradation resulted in the accumulation of acetate, which could slow down the degradation of DMAC. DMAC is degraded by repeated oxidative demethylations leading to the generation of acetamide. The acetamide is further degraded to acetate and nitrate, ultimately resulting in the acetate accumulation and organic nitrogen build-up.

Acknowledgments

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